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Short communication

Association of the bovine leukocyte antigen major histocompatibility complex class II *DRB3*4401* allele with host resistance to the Lone Star tick, *Amblyomma americanum*☆

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Abstract

The MHC of cattle, known as the bovine leukocyte antigen (BoLA) complex, plays an integral role in disease and parasite susceptibility, and immune responsiveness of the host. While susceptibility to tick infestation in cattle is believed to be heritable, genes that may be responsible for the manifestation of this phenotype remain elusive. In an effort to analyze the role that genes within the BoLA complex may play in host resistance to ticks, we have evaluated components of this system within a herd of cattle established at our laboratory that has been phenotyped for ectoparasite susceptibility. Of three microsatellite loci within the BoLA complex analyzed, alleles of two microsatellite loci within the BoLA class IIa cluster (DRB1-118 and DRB3-174) associated with the tick-resistant phenotype, prompting further investigation of gene sequences within the *DRB3* region. *DRB3* is a class IIa gene, the second exon of which is highly polymorphic since it encodes the antigen recognition site of the DR class II molecule. Analysis of the second exon of the *DRB3* gene from the phenotyped calves in our herd revealed a significant association between the *DRB3*4401* allele and the tick-resistant phenotype. To our knowledge, this is the first report of a putative association between a class IIa *DRB3* sequence and host resistance to the Lone Star tick. Elucidation of the mechanism involved in tick resistance will contribute to improving breeding schemes for parasite resistance, which will be beneficial to the cattle industry.
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1. Introduction

Ticks and tick-borne diseases are of veterinary and medical importance worldwide, and have a significant economic impact on the livestock industry with an estimated annual worldwide loss in the range of US$ 18 billion (deCastro, 1997). Tick infestation can have deleterious effects on livestock production traits (Springell, 1983; Mattioli et al., 2000; Jonsson, 2006) and, more importantly, the spread of tick-borne

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diseases, such as babesiosis, anaplasmosis, theileriosis, and heartwater has had a dramatic effect on livestock morbidity and mortality, subsequently affecting the livelihood of farming communities in developing countries (Jongejan and Uilenberg, 2004). Tick control methods have relied primarily on the use of acaricides, but this intensive use has resulted in the development of resistant tick populations (George et al., 2004). One approach to the problem of resistance development is the identification of alternative control technologies such as natural host resistance and vaccination.

Natural host resistance to tick infestation has a genetic basis (Axford et al., 2000), as evidenced by breed-associated differences in susceptibility to ticks (Willadsen, 1980; deCastro and Newson, 1993; Wambura et al., 1998). These differences in the genetic makeup of cattle breeds translate into variable immune responsiveness to diseases and parasites. While it is apparent that the host immune system plays an important role in cattle resistance to tick infestation, the host genes that are involved and the mechanism by which the phenotype is manifested have yet to be elucidated. In an effort to examine what genes are important to parasite resistance in cattle, a herd of calves phenotyped for ectoparasite susceptibility was established at our laboratory with the aid of a local producer. Specifically, individuals were phenotyped for susceptibility to the horn fly, *Haematobia irritans* (Pruett, unpublished), and to the Lone Star tick, *Amblyomma americanum*. Our aim was to evaluate candidate genes within these phenotyped hosts for associations with ectoparasite susceptibility.

The major histocompatibility complex (MHC), known as the bovine leukocyte antigen (BoLA) in cattle, plays an essential role in the regulation of the immune response and genes within the complex have been implicated in resistance or susceptibility to diseases and parasites in ruminants (reviewed in Dukkipati et al., 2006; reviewed in Takeshima and Aida, 2006). In order to examine whether genes within the BoLA region play a role in resistance to tick infestation, we evaluated microsatellite markers within or near BoLA, as well as sequence variations within an exon of a highly polymorphic BoLA gene, for correlation with resistance to infestation by *A. americanum*. Knowledge regarding genetic markers associated with tick resistance will facilitate improved host breeding for parasite resistance, and vaccine development, providing benefits to the cattle industry (Pruett, 1999; Brossard and Wikel, 2004; Kashino et al., 2005).

2. Materials and methods

A. americanum colony ticks used for infestation were routinely maintained at a 12-h light:12-h dark photoperiod, 25 °C, and at ~85% RH. Larval ticks were fed on guinea pigs, while nymphs and adults were fed on cattle.

Data were accumulated for three calf production cycles (2002, 2003, and 2004) from a herd that is comprised of 3 sires (designated B12, B13, and RB), 72 dams of which 5 were progeny of the F_1, and 117 calves of which 5 are F_2 progeny. Two of the three sires (B12 and B13) are siblings that were initially selected for the development of a herd to investigate host resistance to the horn fly, *Haematobia irritans*. B12 and B13 are full sibs from a Red Poll ♀ × Simmental ♂ cross. Semen from the Simmental ♂ was used to artificially inseminate a Red Poll ♀, the flushed embryos from which were separately transferred to surrogate cows. RB, the third sire bull, is of the Simbrah breed. The dams in the herd were commercial cattle of mixed *Bos taurus* and *Bos indicus* influence.

Tick infestations occurred between the months of November and April in open barns with fluorescent lights on a 12-h light:12-h dark cycle. For the initial tick exposure, calves approximately 8 months of age were stanchioned and infested with 500 nymphal ticks, to increase antigenic stimulation, and 30 adult male–female pairs of *A. americanum* ticks. Different life-stages were placed separately under patches. Calves were rested for 2–6 weeks prior to a second challenge infestation with only 30 adult male–female pairs. In each infestation detached, engorged females were collected, weighed individually, and weights totaled (total engorgement weights). Adult female tick feeding performance of the colony ticks was similar in the 3 years of this study, i.e. 85% of adult females fed to repletion in 16 days on initial exposure. Thus, the data from the 3 years were treated as one group for statistical analysis. In total, 101 calves were phenotyped using this experimental scheme. The upper- and lower-quartile total tick second engorgement weights were utilized to designate 25 each of resistant and susceptible calves within our herd, while those calves in the two middle quartiles were not included in our analysis.

Genomic DNA was isolated for every individual within the herd from 10 ml of fresh or frozen whole blood using the Puregene™ Genomic DNA Purification System (Gentra, Minneapolis, MN), and paternity was verified for all calves using the Cattle Parentage Typing System (Applied Biosystems, Foster City, CA). The isolated genomic DNA was archived at our laboratory to

establish a DNA database with correlated ectoparasite susceptibility (phenotype) data for future gene mining studies. B12 and B13, while from separate embryos transplanted to separate surrogate cows, appear to be identical based on analysis of 11 microsatellite loci included as part of the parentage typing system and four microsatellite loci analyzed below.

Oligonucleotide primers were synthesized to amplify four microsatellite loci that map to bovine chromosome 23 (BTA 23), within or near the BoLA complex. The primer pair sequences used were LA54 (DRB3): 5′-GAGAGTTTCACTGTGCAG-3′/LA53: 5′-CGCGAATTCCCAGAGTGAGTGAAGTATCT (Ellegren et al., 1993), RM185F: 5′-TGGCCTGCTTATGCTTGCATC-3′/RM185R: 5′-GAGTTTCCTTTGCATGCCAGTC-3′ (Ihara et al., 2004), MB025F (DRB1): 5′-ATGGTGCAGCAGCAAGGTGAGCA-3′/MB025R: 5′-GGGACTCAGTCTCTCTATCTCTTTG-3′ (Ihara et al., 2004), and BM1815F (DIB): 5′-AGAGGATGATGGCCTCCTG-3′/BM1815R: 5′-CAAGGAGACAAGTCAAGTTCCC-3′ (Ihara et al., 2004). All forward primers were labeled with the 6FAM fluorescent dye (Applied Biosystems) at the 5′ terminus, while all reverse primer sequences were unlabeled.

Bovine genomic DNA was amplified using each primer pair separately, as described above, using approximately 20 ng of genomic DNA as template in a 15 μl reaction consisting of 15 mM Tris–HCl (pH 8.0), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.2 mM dNTPs, 10 pM each primer, and 1U AmpliTaq Gold™ DNA polymerase (Applied Biosystems). Cycling conditions for the LA54/LA53 primer pair consisted of an initial denaturation at 95 °C for 15 min followed by 31 cycles of 94 °C for 45 s, 64 °C for 45 s at 50% ramping speed, and 72 °C for 30 s at 75% ramping speed with a final extension of 72 °C for 10 min. The same cycling conditions were utilized for the remaining three primer pairs with the exception of a 61 °C annealing temperature. PCR-amplified products were analyzed on an ABI Prism 3100/3130xl Genetic Analyzer with the GeneScan-500 ROX size standard (Applied Biosystems), and data was interpreted using GeneMapper™ Software, Version 3.5 or 3.7 (Applied Biosystems).

The exon 2 sequence of *DRB3* was analyzed for each of the 50 phenotyped calves, representing 25 tick-susceptible and 25 tick-resistant individuals, utilizing the PCR-RFLP approach described by van Eijk et al. (1992). Allele assignments were verified by introduction of the PCR-amplified fragments into the pCR4 cloning vector (Invitrogen, Carlsbad, CA) and subsequently sequencing 5–15 clone isolates.

Statistical tests of summarized data were conducted using SigmaStat, Version 3.1 package (Systat Software Incorporated, Richmond, CA). The differences in the frequency of microsatellite alleles and relevant haplotypes, and *DRB3*-Exon 2 alleles at a frequency >0.10 between calves phenotyped as tick-resistant or tick-susceptible were assessed using a two-tailed Fisher exact test.

3. Results

Calves yielding ticks with total second engorgement weights greater than 13.646 g were designated ‘‘susceptible’’ (S), while those lower than 4.302 g were assigned the ‘‘resistant’’ (R) phenotype. All the phenotyped R calves and 16 of the 25 phenotyped S calves were sired by either B12 or B13 (not discernable as described in Section 2), while the remaining 9 phenotyped S calves were sired by the third bull, RB.

A total of four microsatellite loci on BTA 23, three of which lie within the BoLA complex (BM1815, MB025, and DRB3) and one of which lies outside (RM185), were analyzed for possible association with the S and R phenotypes and the resulting frequency of the microsatellite alleles at each locus is summarized in Table 1. No associations were identified between these markers and the S phenotype. However, significant association was observed between the R phenotype and allele 118 of the MB025 (DRB1) locus (DRB1-118; $P = 0.023$) and allele 174 of the DRB3 locus (DRB3-174; $P = 0.001$). No association was observed between the R phenotype and the RM185 or BM1815 loci.

Microsatellite allele haplotypes for the DRB1 and DRB3 loci were deduced because they individually display an association with the R phenotype and are situated near each other on BTA 23. Five individuals of the S phenotype and 18 individuals of the R phenotype had at least one copy of the DRB1-118/DRB3-174 haplotype (data not shown). Evaluation of the haplotypes revealed a significant association between the DRB1-118/DRB3-174 haplotype and the R phenotype ($P = 0.0005$).

The results of the microsatellite analysis suggest there are genes on BTA 23 associated with the R phenotype, especially within the class IIa DRB region. To analyze this further, *DRB3*-Exon 2 alleles were identified for the 50 S/R phenotyped calves using a combination of the PCR-RFLP method (van Eijk et al., 1992) and clone sequencing. The most frequent alleles in the collection of phenotyped calves were **4401*,

Table 1
Allele frequency of four bovine microsatellite loci amplified from calves phenotyped as tick-susceptible (S) or -resistant (R)

(A) DRB3 locus

Allele	S	R	AF[a]	P^b
151	1	2	0.03	–
168	0	1	0.01	–
174	6	21	0.27	0.001*
185	14	7	0.21	0.139
187	1	2	0.03	–
189	3	0	0.03	–
193	9	6	0.15	0.577
194	14	11	0.25	0.645
230	2	0	0.02	–

(B) MB025 locus (BoLA-DRB1)

Allele	S	R	AF[a]	P^b
118	13	25	0.38	0.023*
124	7	6	0.13	0.999
126	2	0	0.02	–
128	14	7	0.21	0.140
133	12	8	0.20	0.454
135	2	3	0.05	–
139	0	1	0.01	–

(C) BM1815 locus

Allele	S	R	AF[a]	P^b
147	24	20	0.44	0.546
149	8	4	0.12	0.357
151	3	8	0.11	0.200
159	2	1	0.03	–
161	4	2	0.06	–
166	9	15	0.24	0.241

(D) RM185 locus

Allele	S	R	AF[a]	P^b
92	1	2	0.03	–
94	5	0	0.05	–
96	1	1	0.02	–
98	14	24	0.38	0.063
100	26	18	0.44	0.158
104	3	4	0.07	–

The loci are (A) DRB3, (B) MB025 [BoLA-DRB1], (C) BM1815, and (D) RM185.

[a] Allele frequency. The total number of individuals = 50 ($N = 25$ per phenotype).

[b] Statistical significance was evaluated for alleles at a frequency >0.10 using a two-tailed Fisher exact test. Significant associations are identified with an asterisk (*).

**1301*, and **0201*, as summarized in Table 2. No homozygotes of exon 2 alleles were observed of the calves studied. *DRB3*4401* was the only allele at a frequency greater than 10% for which a significant association was identified (Table 2), specifically with the R phenotype ($P = 0.011$).

Table 2
BoLA-DRB3 typing of calves that have been phenotyped for tick-susceptibility

Allele	S[a]	R[a]	AF[b]	P^c
*DRB3*4401*	5	18	0.23	0.011*
*DRB3*1301*	12	8	0.2	0.087
*DRB3*0201*	6	4	0.10	0.300
*DRB3*0301*	5	1	0.06	
*DRB3*1001*	3	3	0.06	
*DRB3*0101*	3	3	0.06	
*DRB3*3001*	1	3	0.04	
*DRB3*0501*	3	0	0.03	
*DRB3*4101*	1	2	0.03	
*DRB3*3301*	1	2	0.03	
*DRB3*1101*	1	1	0.02	
*DRB3*4301*	2	0	0.02	
*DRB3*3201*	0	2	0.02	
*DRB3*1701*	1	1	0.02	
*DRB3*1002*	1	0	0.01	
*DRB3*2001*	0	1	0.01	
*DRB3*0801*	0	1	0.01	
*DRB3*1001/1002*[d]	2	0	0.02	
*DRB3*0101/0102*[d]	1	0	0.01	
Total alleles	48[e]	50		

[a] The number of tick-susceptible (S) or -resistant (R) calves with at least one copy of the *DRB3* allele ($N = 25$ per phenotype).

[b] Allele frequency.

[c] A two-tailed Fisher exact test was utilized to evaluate alleles >0.10 in frequency for statistical significance, as identified by an asterisk (*).

[d] Based on PCR-RFLP results.

[e] Maternal allelic contribution could not be verified for two of the S calves.

4. Discussion

Cattle express both innate and acquired immunological resistance to tick infestation as a result of natural exposure (Willadsen, 1980; Wikel, 1996). Evidence of genetic variation in resistance to disease and parasites has focused research efforts on the identification of loci that are responsible for the susceptible or resistant phenotype (Spooner, 1997), with an interest in incorporating those genes with desirable production traits.

The MHC of cattle, known as BoLA, is located on bovine chromosome 23 and variations in classes I and II genes may influence cattle immune responses (Takeshima and Aida, 2006). The class II molecules, encoded by class II genes, bind processed peptides from extracellular antigens and present them to epitope-specific $CD4^+$ T lymphocytes (Abbas and Lichtman, 2003). Class II genes are of interest, as they have been associated with resistance and susceptibility to animal diseases (Amills et al., 1998) and with cattle immune responsiveness (Rothschild et al., 2000)

In the current study, we identified two microsatellite alleles (DRB1-118 and DRB3-174) that associate with the designated R phenotype, suggesting that the region in which the BoLA class IIa genes are located is of particular interest. This was strengthened by the significant association of the DRB1-118/DRB3-174 haplotype with the R phentoype, and by an absence of association with the RM185 locus, which lies outside of the BoLA complex, and the BM1815 locus, which is located between the BoLA class IIa and IIb clusters. Class IIb genes are located in a separate cluster from the IIa genes, are not highly polymorphic, and have not been implicated in disease or parasite resistance in cattle (Takeshima and Aida, 2006). Acosta-Rodriguez et al. (2005) analyzed the same microsatellite loci for association with host resistance to the Southern cattle tick, *Boophilus microplus*, and reported three alleles associated with tick-susceptibility but none associated with the non-susceptible phenotype. This could be attributed to the considerable differences between the genetic makeup of the cattle, the infestation scheme, the tick species, and the phenotype designation criteria that were utilized in this study and that of Acosta-Rodriguez et al. (2005).

The second exon of the *DRB3* gene, which is located within the BoLA class IIa cluster and is highly polymorphic, encodes the antigen recognition site that binds to a wide array of processed peptides (Sigurdardottir et al., 1991; van Eijk et al., 1992). As such, significant associations between alleles of *DRB3*-Exon 2 and susceptibility to bovine dermatophilosis (Maillard et al., 2002), resistance to persistent lymphocytosis (Zanotti et al., 1996), and susceptibility to mastitis (Sharif et al., 1998; Park et al., 2004) have been reported in the literature. Analysis of *DRB3*-Exon 2 alleles in our herd revealed that **4401* and **1301*, representing 43% of the total alleles (Table 2), were the most frequent. This was not unexpected since they represent the paternal contribution from B12 and B13, sires for 82% of the phenotyped calves. However, *DRB3*4401* was significantly associated with the R phenotype ($P = 0.011$) while, in direct contrast, *DRB3*1301* was not associated with either phenotype ($P = 0.087$).

Ellegren et al. (1993) reported an association between length polymorphisms of the DRB3 microsatellite marker and *DRB3* alleles encoded by exon 2. Data from this study support the diagnostic value of the microsatellites, as all tick-resistant individuals with the DRB3-174 allele also had the **4401* exon 2 allele. To our knowledge, this represents the first report of a putative association between a BoLA class II *DRB3* allele and host resistance to the Lone Star tick.

The **4401* allele was reported fairly recently from Gudali zebu cattle (Maillard et al., 2001) and has since been identified within Jersey and Japanese Black cattle (Takeshima et al., 2003); the allele was absent from surveyed Holstein and Japanese Shorthorn breeds (Takeshima et al., 2003). To date, its frequency in cattle breeds has not been thoroughly analyzed. The data presented here associating **4401* with the R phenotype prompts further investigation into the frequency of the allele and encourages additional research into its potential contribution in explaining between-breed differences to tick infestation. Overall, this study provides insight into one of the many possible host–parasite interactions that may play a role in the elaborate mechanism that defines the tick-resistant phenotype.

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